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DRI-PR 93-01
UGUST 1993

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D. W. TURNER

E. D. PEDERSON

B. L. LAMBERTS

Naval Medical Research and Development Command
Bethesda, Maryland

NAVAL DENTAL RESEARCH INSTITUTE
BUILDING 1-H
GREAT LAKES, ILLINOIS 60088-5259

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Research Progress Report NDRI-PR 93-01
Work Units 61153N MR04120.002-0445 and
MR04120.002-0051
Naval Medical Research and Development Command
National Naval Medical Center
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

Approved and released by:

S. A. RALLS
Captain, Dental Corps
United States Navy
Commanding Officer

A sensitive method for the detection of immune complexes in human gingival crevicular fluid

D. W. Turner[1], E. D. Pederson[2] and B. L. Lamberts[2]

[1]Department of Periodontics, School of Dentistry, Northwestern University, Chicago, Illinois 60611-2909; [2]Naval Dental Research Institute, Building 1-H, Great Lakes, Illinois 60088-5259 USA

Turner DW, Pederson ED and Lamberts BL: A sensitive method for the detection of immune complexes in human gingival crevicular fluid. J Clin Periodontol 1992; 19: 601–603.

Abstract. An assay for immune complexes [ICs] in gingival crevicular fluid [GCF] could be potentially useful to ascertain the risk of periodontal disease activity at specific oral sites. This paper describes an ELISA-based method sufficiently sensitive to measure ICs in GCF samples at levels below 1 μg/ml. Static GCF samples taken from 5 adults showed IC levels ranging from 5 to 166 μg/ml. Additional tests of 10 GCF samples from 1 adult were conducted with complement components and indicated that either C1q or rabbit anti-human C3d was suitable as a capture agent in the assay procedure. Further application of this assay may help to assess the usefulness of IC levels in GCF samples as possible diagnostic indicators of periodontal disease activity.

Key words: host response; immune complexes; gingival crevicular fluid; ELISA

Accepted for publication 6 April 1992

Several investigators have examined the possible role of immune complexes [ICs] in human periodontal diseases (Genco & Krygier 1972, Toto et al. 1978, Clagett & Page 1978). The conclusion from these earlier studies was that, in gingivitis and chronic adult periodontitis, deposits of ICs and complement are not major characteristics of the pathogenesis of the diseases (Genco & Slots 1984).

Recently, more refined histologic and immunologic methodologies have been developed along with commercially available immunoreagents to various putative inflammatory factors. Their use has shown that antigen-antibody-complement complexes are localized in dental periapical inflammatory lesions (Johannessen et al. 1983). More recently, immunoglobulins have been demonstrated together with various activated complement components within and adjacent to vessel walls, indicating that IC-mediated vasculitis is involved in the pathogenesis of inflammatory periodontal lesions (Nikolopoulou-Papaconstantinou et al. 1987).

The objective of this study was to develop a sensitive method for assaying complement-fixing ICs, to determine whether ICs could be detected in static gingival crevicular fluid (GCF). The presence of ICs in GCF might be due to high IC levels in and adjacent to periodontal tissues. Such IC levels could reflect potential or active disease at specific sites.

Material and Methods

Patients at the Northwestern University Dental School Clinic provided the GCF samples used in this study. The patients were categorized as demonstrating types II and III periodontitis according to the American Dental Association classification. The patients for the first part of the study included two of type II (females, ages 39 and 46 years), and two of type III (one male, age 49, and one female, age 47 years). 5 samples were obtained from each patient, using apparently healthy 2–4 mm probing-depth periodontal sites for the type II subjects and apparently diseased 5–7 mm probing-depth sites for the type III subjects. For the 2nd part of the study, 12 samples were obtained from 1 type III patient (female, age 48 years).

The GCF samples were collected on Periopaper® strips and fluid volumes were estimated (0.034–1.600 μl) with a Periotron 6000 (PRO FLOW, Inc., Amityville, NY 11701). Each sample was stored dry in a screw-cap vial at −70°C prior to assay. ICs were eluted from the strip into a final volume of 300 μl by the method of Ebersole (1984), modified as follows: 50 μl of phosphate-buffered saline (Voller et al. 1976), pH 7.2, containing 1% Triton X-100 and 0.02% sodium azide, was added to the strip in a microcentrifuge tube, and, after 5 min, the sample was centrifuged for 1 min at $500 \times g$. This step was repeated 5 times, but with intervals of 10, 10, 15, 20, and 30 min, respectively, after each subsequent addition of eluent, and with centrifugation in the final step at $2000 \times g$.

The IC assay was adapted from the ELISA system of Singh & Tingle (1982), using heat-aggregated human IgG (AHG) as standard. The assay involved the binding of biotinylated anti-human IgG to IC or AHG, immobilized by a capture agent. 2 capture agents, C1q and rabbit anti-human C3d, were examined. After avidin-conjugated alkaline phosphatase was introduced as a label, the substrate amplification system of Carr et al. (1987) was employed to enhance the sensitivity of the procedure. The assay was performed as follows:

The capture agent, C1q (Calbiochem) or rabbit anti-human C3d (DAKO Immunoglobulins, Santa Barbara, CA), was bound to polystyrene plates by overnight incubation at 4°C. The plates were blocked by incubating with 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), pH 7.2, at 37°C for 2 h, then at 4°C overnight. After washing with

PBS-T, plates were stored at −70 C until used. PBS-T was used in all of the plate washings for the assay.

The samples and standards were added to each plate in 25-μl volumes, usually in triplicate, and incubated at 37 C for 60 min. After plate washing, 25 μl of a 1:50,000 dilution of biotinylated goat anti-human IgG (Zymed Labs, San Francisco, CA) was added to each well and the plate was incubated at 37 C for 40 min. After plate washing, 25 μl of a 1:24,000 dilution of avidin-conjugated alkaline phosphatase (Zymed Labs) was added to each well, the plate was incubated at 37 C for 40 min, and was again washed.

The substrate amplification system of Carr et al. (1987) was used to test for alkaline phosphatase activity, as summarized below.

(1) The primary substrate, 40 μl of 0.2 mM NADP (Sigma Chemical Co., St. Louis, MO) in 50 mM diethanolamine buffer, pH 9.5, was added to each well and the plate incubated at 24 C for 15 min.

(2) The secondary substrate, 110 μl of a mixture of ethanol, alcohol dehydrogenase, diaphorase (2.1–2.4 units/ml), and p-iodonitrotetrazolium violet (Sigma) in 25 mM sodium phosphate buffer, pH 7.2, was added to each well. The plate was then read immediately on a model 2550 spectrophotometric plate reader (Biorad Laboratories, Richmond, CA) at 492 nm.

IC levels were calculated as AHG equivalents based on the standard curve generated for each assay plate.

Results and Discussion

The initial assays, with C1q as capture agent, were performed on GCF samples from the first group of four patients, as shown in Table 1. These representative data demonstrated that the assay was sufficiently sensitive to measure IC levels in GCF samples.

Additional exploratory tests were conducted using rabbit anti-human C3d as capture agent in place of C1q. Table 2 presents comparative data on the use of C1q and the anti-human C3d as capture agents for assays of IC levels in GCF samples. Analysis of the data, arranged in ascending order of IC levels with use of the C1q, indicated a correlation of 0.995 for the 2 methods ($p < 0.001$). Standard curves yielded linear plots for both agents, with $r = 0.874$ for C1q and $r = 0.995$ for the anti-human C3d over the same ranges. These findings indicate that comparable results can be obtained with the two agents; however, the use of the anti-human C3d would seem to be preferable in view of its lower cost and greater commercial availability.

Although the data of Table 1 showed the mean IC levels in GCF samples to be higher in 5–7 mm probing-depth sites from type III subjects compared to 2–4 mm probing-depth sites from type II subjects, the data on Table 2 from one type III subjects showed wide variations of IC levels in relation to probing depths.

Aside from past work, cited earlier, there is a paucity of data to suggest that ICs exist in gingival tissues. The detection of ICs in GCF may indicate that (a) the complexes elute as a serous exudate out of tissue after being formed, (b) the complexes are transported out of tissue by cells that possibly lyse in the GCF or (c) IgG antibodies and serum complement components diffuse from the tissues and form complexes in the GCF with antigens released from subgingival plaque. The last possibility would seem to be most likely, since large quantities of bacteria and specific immunoglobulins exist in gingival pockets.

Low molecular-weight complement fragments such as C3a could reenter the tissue and stimulate mast cells to degranulate, thus increasing vascular permeability. Factor C5a, generated in GCF, could be chemotactic for neutrophils located in the tissues. The migration of the cells through the tissues in response to chemotactic stimuli could generate an inflammatory response when lysosomal enzymes are released along the migratory route. Furthermore, ICs located in tissue or in GCF could activate neutrophils via receptor-ligand binding of the Fc portion of IgG to the neutrophil Fc receptor.

Although it can be presumed that this assay detects soluble ICs in GCF, it would appear likely that insoluble ICs could also be detected. This aspect of ICs has not been examined in the present study.

The elution method employed to recover ICs from filter strips by the use of PBS and 1% Triton X-100 was adopted after several preliminary tests. IC recovery rates from these tests were as follows: PBS alone, 70–75%; PBS and 1% Tween 20, 80–85%; PBS and 1% Triton X-100, 95–100%.

The method we have described utilizes minute quantities of samples and reagents, and demonstrates a relatively simple ELISA procedure for detecting ICs. Large numbers of samples can be processed rapidly. However, the signifi-

Table 1. Levels of immune complexes in GCF samples from 2 type II and 2 type III subjects

2–4 mm probing-depth sites from type II subjects IC (μg/ml)	5–7 mm probing-depth sites from type III subjects IC (μg/ml)
5	74
29	100
40	120
52	125
58	125
59	132
65	139
67	145
81	151
129	166
mean 58.5	mean 127.7
SD 32.8	SD 26.2

Table 2. Comparative IC levels in GCF samples from 1 type III subject measured by use of C1q and anti-human C3d as capture agents

Sample	Tooth no.	Probing depth	IC levels (μg/ml) C1q	C3d
1	9	3	0*	0*
2	8	3	0*	8
3	3	5	0*	15
4	11	5	1	5
5	6	5	3	6
6	10	3	22	29
7	19	5	28	28
8	7	3	38	42
9	20	7	49	46
10	14	5	103	103
11	30	4	106	111
12	29	6	112	113
		mean	38.5	42.2
		SD.	44.4	42.9

* < 1 μg/ml

cance of ICs in GCF in relation to periodontal pathology has not been established. Once this has been accomplished, the method could be applied to obtain longitudinal data on a much larger group of subjects. Such data could determine whether IC levels in GCF would provide a significant parameter in a profile to indicate active periodontal diseases.

Acknowledgements

The authors wish to thank Ms. M. Portis for assistance with manuscript preparation. This research project was supported by the Naval Medical Research and Development Command by Projects 61153N MR04120.002-0445 and MR04120.002-0051. The opinions expressed herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or the Naval Service at large. The use of commercially available products does not imply endorsement of the products or preference over similar products on the market.

Zusammenfassung

Eine sensible Methode zum Nachweis von Immunkomplexen im Zahnfleischsekret des Menschen. Eine kurze Mitteilung

Zur Ermittlung eventuell vorhandener parodontaler Krankheitsaktivität in bestimmten oralen Regionen, könnte ein Verfahren zum Nachweis von Immunkomplexen (ICs = immune complexes) im Zahnfleischsekret (GCF = gingival crevicular fluid) positive Möglichkeiten eröffnen. In der vorliegenden Veröffentlichung wird eine Methode beschrieben, die sich auf das ELISA-Verfahren stützt. Die Empfindlichkeit dieser Methode reicht aus, um in GCF-Abstrichen ICs registrieren zu können, die unter 1 μg/ml liegen. Bei statischen, an 5 Erwachsenen entnommenen GCF-Stichproben, wurden zwischen 5 und 166 μg/ml liegende IC-Niveaus registriert. Darüber hinaus wurden 10, bei einem Erwachsenen entnommene Stichproben, mit Komplement-Komponenten untersucht. Es zeigte sich, daß sich bei diesem Nachweisverfahren entweder C1q oder anti-humanes Kaninchen C3d als catch-up Antikörper eignete. Eine weitere Anwendung dieser Nachweismethode kann zur Erkundung der Fragestellung beitragen, ob IC-Niveaus in GCF-Abstrichen als denkbare diagnostische Indikatoren für die Aktivität parodontaler Krankheiten infrage kommen können.

Résumé

Méthode sensible pour détecter les complexes immuns dans le fluide gingival humain

Une mesure des complexes immuns (ICs) dans le fluide gingival (GCF) pourrait posséder le potentiel de servir à constater la présence d'un risque de parodontopathie active dans des sites buccaux spécifiques. Le présent article décrit une méthode basée sur l'ELISA et suffisamment sensible pour mesurer dans des échantillons de GCF les ICs à des niveaux inférieurs à 1 μg/ml. Des échantillons statiques de GCF recueillis chez 5 adultes mettaient en évidence des niveau d'IC allant de 5 à 166 μg/ml. Des tests supplémentaires ont été faits avec les éléments du complément sur 10 échantillons de CGF provenant d'un adulte et indiquaient qu'on pouvait utiliser soit C1q soit le C3d anti-humain de lapin comme agent de capture dans cette méthode de mesure. L'application ultérieure de cette mesure peut contribuer à établir la valeur de l'utilisation des niveaux d'IC dans les échantillons de CGF comme indicateurs diagnostiques de l'activité de la maladie parodontale.

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Address:

Donald W. Turner
Naval Dental Research Institute
Building 1-H
Great Lakes, IL 60088-5259
USA

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) A SENSITIVE METHOD FOR THE DETECTION OF IMMUNE COMPLEXES IN HUMAN GINGIVAL CREVICULAR FLUID		5. TYPE OF REPORT & PERIOD COVERED
		6. PERFORMING ORG. REPORT NUMBER NDRI-PR 93-01
7. AUTHOR(s) D. W. TURNER, E. D. PEDERSON AND B. L. LAMBERTS		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Dental Research Institute Building 1-H Great Lakes, IL 60088-5259		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61153N MR04120.002-0445 AND MR04120.002-0051
11. CONTROLLING OFFICE NAME AND ADDRESS Naval Medical Research and Development Command National Naval Medical Center, 8901 Wisconsin Ave. Bethesda, MD 20889-5606		12. REPORT DATE August 1993 13. NUMBER OF PAGES (3)
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) Chief, Bureau of Medicine and Surgery Navy Department 2300 E Street NW Washington, DC 20372-5300		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

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18. SUPPLEMENTARY NOTES

Journal of Clinical Periodontology 1992; 19: 601-603

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Host Response, Immune Complexes, Gingival Crevicular Fluid, ELISA

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An assay for immune complexes [ICs] in gingival crevicular fluid [GCF] could be potentially useful to ascertain the risk of periodontal disease activity at specific oral sites. This paper describes an ELISA-based method sufficiently sensitive to measure ICs in GCF samples at levels below 1 μg/ml. Static GCF samples taken from 5 adults showed IC levels ranging from 5 to

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S/N 0102-LF-014-6601

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166 μg/ml. Additional tests of 10 GCF samples from 1 adult were conducted with complement components and indicated that either C1q or rabbit anti-human C3d was suitable as a capture agent in the assay procedure. Further application of this assay may help to assess the usefulness of IC levels in GCF samples as possible diagnostic indicators of periodontal disease activity.